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# GROWTH AND DEVELOPMENT OF THE LABORATORY CULTURED SEA URCHIN[1]

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The sea urchin embryo has played a key role in embryological studies for almost a century. Derbès first described the developmental process (though not with complete accuracy) in 1847. In 1876 the eggs were used in Hertwig's experiments demonstrating the role of sperm in fertilization, and in 1891, Driesch used sea urchin eggs in his experiments showing indeterminate cleavage. In recent years the sea urchin egg has become particularly prominent in the study of both the morphology and biochemistry of early development. (See reviews by Gustafson and Wolpert, 1963; Gross, 1967; Davidson, 1968.)

Probably more is now known about the early development of the sea urchin than about any other organism. The reasons for this, other than the fact that early echinoderm and vertebrate development are similar, are primarily technical. The animals are easy to obtain on almost any sea coast, they spawn readily and yield large numbers of eggs. The eggs complete meiosis in the ovaries and therefore can be fertilized immediately after spawning. Fertilization is easily accomplished simply by adding sperm to an egg suspension. Development usually follows with good synchrony and with minimum care. These eggs readily take up a large number of chemicals of biological interest, and they have the further advantage of being small and containing much less yolk than either frog or chicken eggs.

One major disadvantage in the use of sea urchins has been the extreme difficulty in raising the larvae beyond plutei to adults and thus obtaining a second generation. This has seldom been done, and never as a useable laboratory procedure. (See Harvey, 1956 for the relevant references.) If the larvae could confidently be raised, it would then be possible to apply genetic techniques to the study of sea urchin development. This, along with the other advantages these eggs offer, would make them almost ideal material to use in unraveling the developmental process.

This paper reports the first steps in that direction. It is an outline of the techniques for raising sea urchins in the laboratory, and a description of the general features of the developmental stages. The techniques have now been developed to the point where large numbers of urchins can be taken from egg to egg. Though the procedure is not yet as simple as raising *Drosophila*, it is practical.

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## Life Cycle of the Sea Urchin

The life cycle of the sea urchin can be divided into six more-or-less distinct phases. (1) The fertilized egg, (2) development through blastula and gastrula to pluteus, at which time egg nutrients are usually consumed, (3) growth and development of the feeding pluteus to a mature larva, (4) development of the embryonic urchin inside the growing larva, (5) metamorphosis, and (6) growth of the young urchin to a reproductive adult. Most other Echinoderm groups develop in a similar way.

This sequence is not a smooth continuum with the structures of each stage giving rise to those of the next. The fourth one is particularly unusual. During this period the urchin, or more accurately the ventral half of the urchin, grows almost as a parasite within the larva. Most of the urchin is derived from a combination of the middle portion of the left hydrocoel and the overlying ectoderm (MacBride, 1903). Few, if any, of the larval organs give rise to comparable organs in the adult. The urchin develops its own mouth and anus, most if not all, of its internal organs, spines, *etc.* The larva comes close to being little more than a source of nutrient and protection. A similar type of embryological development is found in the insects and Nemertines. In both of these there are imaginal discs which give rise to portions of the adult. These discs also have close to an independent existence. The Echinoids differ, however, since one equivalent to a disc ultimately gives rise to the whole urchin.

### *The larvae*

*Culture Methods:* The larvae of *Arbacia punctulata, Lytechinus pictus,* (Pacific Bio-Marine Supply Co., P. O. Box 536, Venice, California 90291), *Lytechinus variagatus,* (Gulf Specimen Co., Panacea, Florida 32346), *Strongylocentrotus purpuratus* and *Echinometra mathaci,* have all been raised. If several conditions are met, any of these larvae can readily be grown to maturity (*i.e.*, up to metamorphosis) in the laboratory. The proper food is most important. Larval concentration and agitation must also be controlled. Both larvae and urchins can be grown in either filtered sea water or a synthetic salt mixture such as *Instant Ocean,* (Aquarium Systems Inc., 1450 E. 289 Street, Wickliffe, Ohio), but growth has been consistently better in sea water.

The type of food organism is critical and out of 14 algal species tried, using either *Arbacia punctulata* or *Lytechinus pictus* as the test organism, only three were found to be satisfactory. These were species of *Dunaliella, Rhodemonas,* and *Pyranimonas.* All three are flagellated algae. The algae that would not serve as food were: *Amphidinium operculayum, Coccolithus huxleyi, Cryptomonas, Cyclotella nana, Cylindrotheca closterium, Eutreptiella, Isochrysis galbana, Melosira nummuloides, Monochrysis lutheri, Nitzschia brevirostris, Phacodactylum tricornatum.* No diatom has been found that alone could serve as food. All algae were grown in pure culture using half strength Guillard's medium (Guillard and Ryther, 1962). The larvae of *Arbacia* grew well on *Dunaliella tertiolecta. Lytechinus pictus, L. variagatus, S. purpuratus* and *E. mathaci* developed better on a diet of an alga designated 3C by Guillard and tentativelly identified as a species of *Rhodemonas. Lytechinus pictus,* and possibly the other species as well, will also

develop on a species of *Pyramimonas* (designated LB 997) that was obtained from Dr. John West, University of California, Berkeley. The concentration of algae used depends on the stage of larval development, with earlier stages being fed much less than older. The larvae were usually fed once a day and given the amount of algae they would consume in 24 hrs or, in the case of young plutei, about 3000 algae per ml. No attempt was made to illuminate the larval cultures or otherwise maintain the algae. Prior to use the algae were centrifuged from their culture media and resuspended in sea water.

If mixtures of algae were used a larger number of species would probably be applicable. The natural conditions in the ocean would also be more closely approximated. However, from an experimental point of view, a single species offers a number of advantages. Maintenance of the algae is easier, feeding is simplified and any nutritional studies or radioactive labeling experiments can be better controlled.

The maximum number of larvae that can be cultured in a given volume of water depends on their stage of development. One individual per milliliter represents a comfortable maximum for mature larvae.

Some form of agitation is usually necessary during larval growth. This prevents a number of potential ills. In still water the plutei of some species tend to remain near the surface and often stick there and die. *Lytechinus pictus* is particularly susceptible to this. About half way through development there is the opposite tendency and the larvae will stay near the bottom. Here they can become trapped in debris. All this can be prevented by some form of gentle stirring. A simple apparatus for doing this is illustrated in Figure 1.

This is essentially a magnetic stirrer, but instead of placing the stirring magnets above the drive magnet, they are placed laterally. This permits a large number of stirrers to be driven simultaneously by one motor. The apparatus illustrated in Figure 1 is 20 inches high and 18 inches in diameter. The bottom shelf rests on ball bearings which allow the shelves to be rotated. Three 6 by $\frac{3}{4}$ by $\frac{1}{4}$ inch Alnico V magnets are mounted on a center shaft that is driven by a 25 RPM motor. The larvae are grown in $3\frac{3}{4}$ inch diameter by $2\frac{3}{4}$ inch polystyrene dishes (no. 42F, Tristate Plastic Molding Company, Henderson, Kentucky). Two holes approximately $\frac{3}{32}$ of an inch in diameter are drilled in the lids of the dishes, one near the edge, the other in the center. Both serve to ventilate the culture. The center hole also holds the axle of the stirrer.

An assembled stirrer is illustrated in the insert at the bottom of Figure 1. The stirrer floats inside the culture dish and consists of a small circular ferrite magnet $\frac{1}{2}$ by $\frac{3}{8}$ inches in diameter (other small magnets can work equally well) cemented inside two lids of 35 × 10 mm polystyrene petri dishes (Falcon Plastics Company, Rochester, New York). A piece of monofilament nylon fish line (approximately 20 pound test) is cemented in the top to serve as an axle. Two paddles are attached to the bottom. The particular paddle design shown, tends to minimize contact between larvae and paddle. The paddles should have a total area of about 2 $cm^2$ for satisfactory operation.

An alternative method is to agitate the culture by gently bubbling air through it, but this generally slows development and reduces the length of larval spines. Of the species that have been raised, *Arbacia* is the only one that grows well without agitation. In fact, it can even be raised in test tubes.

FIGURE 1. Culture apparatus, with culture dishes. Insert shows construction of the magnetic stirrer. See text for description.

The five species that have been worked with are not all equally adaptable to laboratory conditions. *Strongylocentrotus purpuratus* requires a temperature of 15° C or lower and therefore necessitates a cold room or water bath. *Arbacia, L. pictus, L. variagatus* and *E. mathaei* will all grow at room temperature (22–24° C). *Lytechinus variagatus* is the least hardy of the four. *Arbacia* is probably the easiest to raise, but the young urchins tend to hold on to any surface with tenacity and are difficult to transfer without breaking off their tube feet. The urchins are also less hardy than their larvae. The larvae of *E. mathaei* are smaller than those of the other species and this creates some difficulties, particularly in handling. The young urchin is also difficult to raise. Therefore most of what will be reported here will be results obtained with *L. pictus*. These larvae are not difficult to raise and the young urchins grow well under laboratory conditions.

*Larval Development:* The eggs of *L. pictus* are obtained by injection of 0.1–0.2 ml of a fresh 0.1 molar acetylcholine-sea water solution (Hinegardner, 1967).

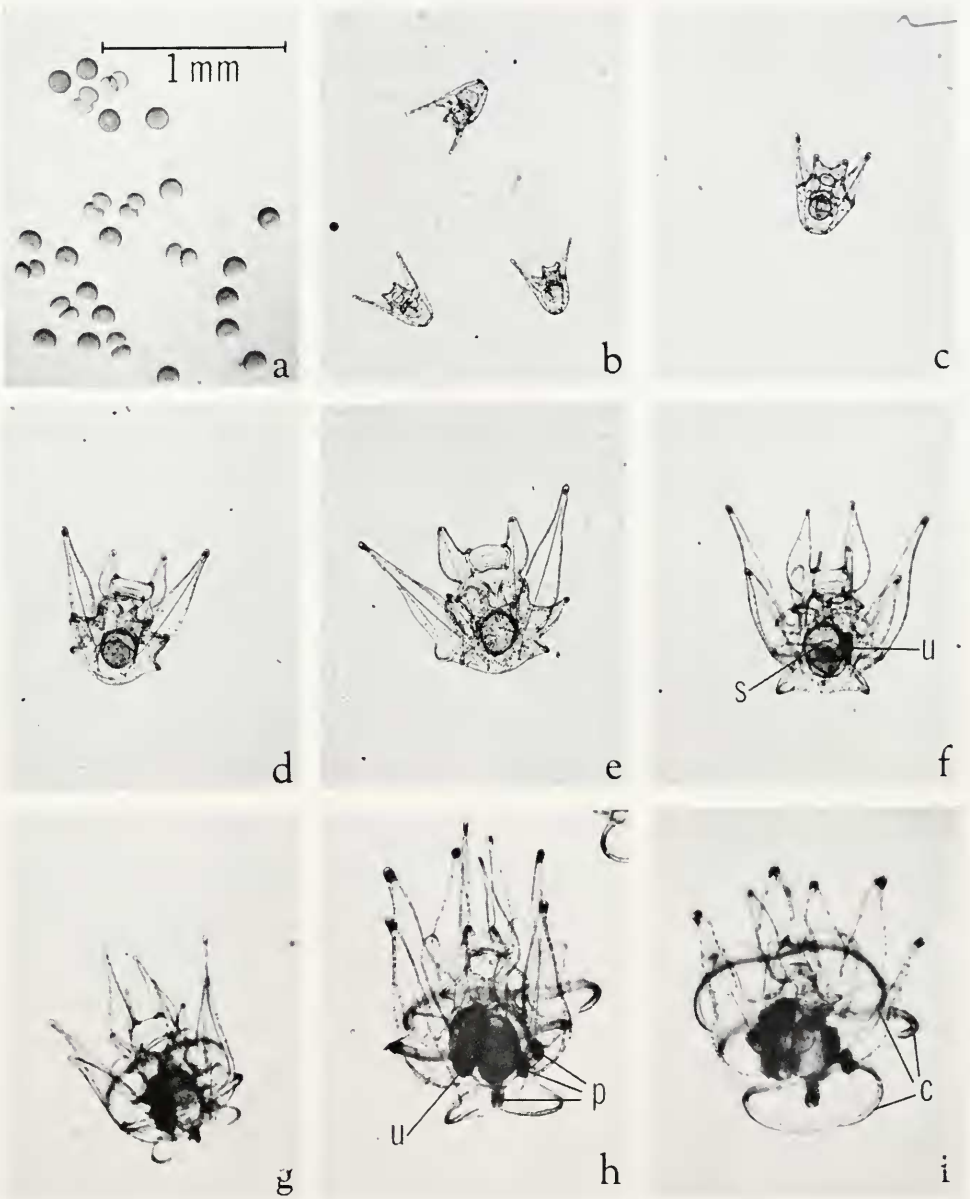


FIGURE 2. Development of the larva of *Lytechinus pictus* from fertilized egg to maturity at 24° C; (a) One and two cell embryos, (b) Pluteus, 2 days old, (c) 4 days, (d) 7 days, (e) 8 days, (f) 11 days (because of orientation the developing urchin appears on the right side), (g) 13 days, (h) 19 days, (i) 26 days. Abbreviations are: c—ciliary bands or epaulets, p—pedicillaria, s—stomach, u—urchin. All pictures are at the same magnification.

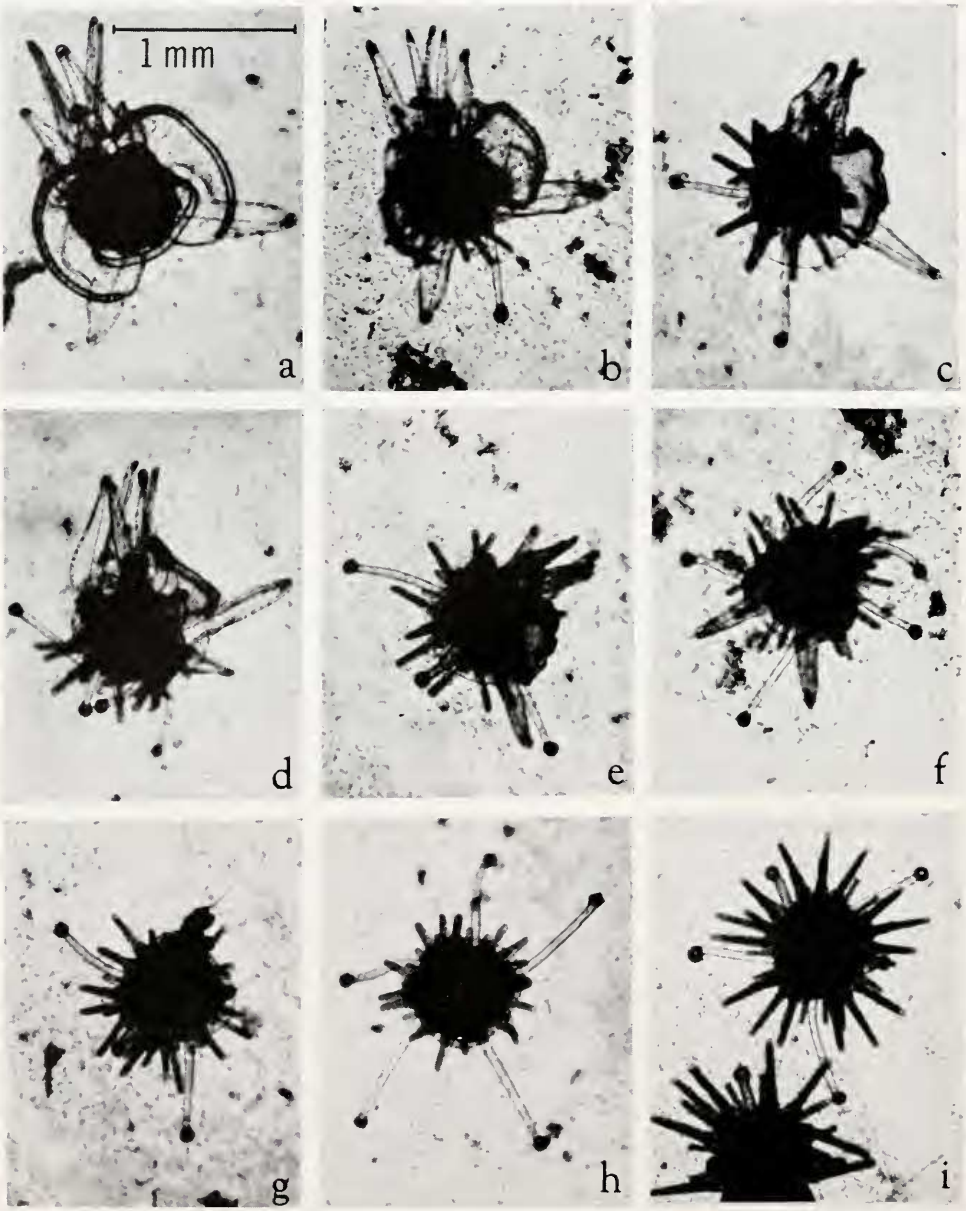


FIGURE 3. Stages in the metamorphosis of *Lytechinus pictus* larvae; (a) 1 minute after adding to the appropriate substrate, (b) 6 minutes, (c) 9 minutes, (d) 11 minutes, side view showing relation of larval structures to the emerging urchin, (e) 12 minutes, (f) 15 minutes, (g) 37 minutes, larval spines are visible in upper right, (h) 80 minutes, (i) 27 hours. All pictures are at the same magnification.

The adult urchins are more apt to survive after this treatment than after the usual 0.5 molar KCl injection or electrical stimulation. The eggs are fertilized and developed at room temperature to early pluteus in the usual way (Costello *et al.*, 1957; Tyler and Tyler, 1966; Hinegardner, 1967). In about two days the plutei are able to feed and, if conditions are close to optimum, they will reach maturity in a month or less.

Figure 2 illustrates larvae at various stages of development. The major morphological changes occurring during this period, aside from overall increase in size, are: (1) The appearance of four additional arms for a total of eight (compare Fig. 2c with Fig. 2h). (2) The formation of heavy ciliary bands which have been called epauletes, and which are most easily seen in Figure 2i. (3) The differentiation of the left hydrocoel and overlying ectoderm into the urchin tube feet primordia (Fig. 4a-c). This is followed by (4) the appearance of tooth and spine primordia and (5) urchin growth and development, primarily of the ventral half. Along with this, there is (6) the development of the three pedicellariae, one posterior and two on the right side of the larva (Fig. 2h). Finally (7) there is metamorphosis of the larva into a small urchin (Fig. 3). The development of the dorsal half and adult internal organs then begins.

Morphological details of the various stages up to metamorphosis have been described by MacBride (1903) for *Echinus esculentus*. A general description of larval development is also given in Kumé and Dan (1968).

Providing the larvae are given resonable care, about 80% of the plutei can be raised to mature larvae. It is difficult to determine what fraction of the non-survivors died from genetic or congential defects, but it would seem that the survival rate is close to maximum.

### *Metamorphosis*

*Loss of larval form.* The physiological aspects of metamorphosis are not yet well undertood, but the visible changes that occur during this process have been followed in detail and they are illustrated in Figure 3. The first event is the settling of the larva on its left side on to an appropriate substrate. A surface covered with a mixture of algae and bacteria can induce this response. Within a minute, the anterior portion of the larva, and the arms, flex sharply toward the larva's right side (compare Fig. 3a to the normal larva in Fig. 2i). This exposes the left side of the larva and the tube feet of the young urchin to the substrate.

The tissue surrounding the urchin is then drawn up and the urchin spines appear. This is accompanied by the collapse of the larval tissue on to the top of the urchin (Fig. 3b-3g). Within about an hour (3g and h), all that remains of the larva is a lump of tissue on top of the young urchin. A few naked larval spines may extend out from it (Fig. 3g) but these are lost in a few hours. Over the next 24 hours the spines of the urchin greatly elongate (Fig. 3i).

Metamorphosis is not an obligatory stage. If the larvae are kept in clean containers they will usually not metamorphose. Instead, they continue to feed, but they grow little if at all. After two months evidence of deterioration becomes apparent and eventually they die. The maximum life span of well fed larvae is probably about four months. Their ability to metamorphose is almost com-

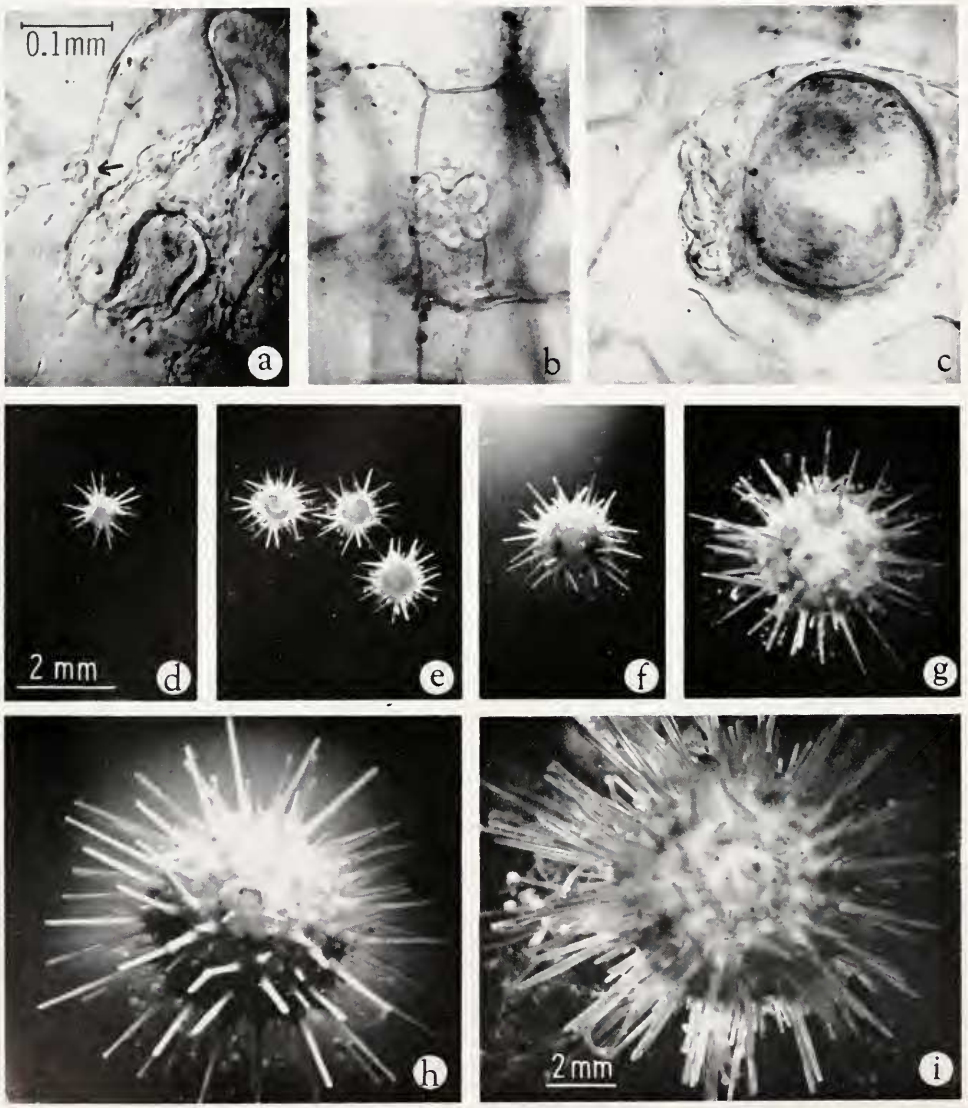


FIGURE 4. Stages in the development of the urchin; (a) 7 day old larva, arrow indicates the invaginating ectoderm; the left hydrocoel is just to the right of arrow and the gut is in the right center of the field; (b) A 9 day larva, showing the beginning of the five tube feet; this larva is rotated 90° to (a) and (c), (c) lateral view of the developing urchin in an 11 day larva; (d) 1 day old urchin; (e) 9 day urchin; (f) 27 day; (g) 71 day; (h) 117 day; (i) 208 day. (a) to (c) are taken through Nomarski optics and are at the same magnification, (d) to (h) are also at the same magnification.

pletely lost at the end of about two months. However if the larvae are underfed, this slows development and the life span can be greatly increased.

The events described here, apply to all the urchin species that have been examined. In a more general way they also apply to other echinoderm classes. Mature starfish and brittle star larvae have been obtained from plankton tows. Their metamorphosis has also been observed and it appears to follow the basic features of the pattern outlined here.

*Development of the urchin.* Figures 3i and 4d illustrate the young urchin. With one exception, the urchins of all the species that have been examined are very similar. However, the young *Arbacia,* which belongs to a different suborder, looks quite different (see Harvey (1956). It has 15 paddle-like spines and looks almost like a flower. The other species have 20 spines. These are cylindrical and arranged in five groups of four. In all species there are three pedicellaria which developed in the larvae and are now on the urchin's dorsal surface.

The young urchin has neither a mouth nor anus and according to MacBride (1903) no gut either. In terms of formal organs, it is little more than half an urchin; the ventral half. There is half a test, 20 ventral spines, five ventral tube feet and the five teeth. At this stage the dorsal half is essentially a rounded lump of larval tissue punctured by the three pedicellaria. The dorsal organs appear to develop out of this tissue. For the first two days the larval tissue can easily be picked off the urchin. If this is done the urchin will still continue to crawl about for a number of days afterward. The digestive system, and probably other internal organs appear at about four or five days. The urchin then begins to feed. This marks the end of the metamorphic period.

*The urchin*

*Growth:* A number of stages during the growth of *Lytechinus pictus* are shown in Figure 4. The young urchin begins to grow after it is 8–10 days old. Along with size increase, there is an increase in the number of spines and tube feet. At a shell diameter of 2 mm the madreporite begins to develop. The gonadopores appear at an age of about two months and a shell diameter of 3.2 mm. Gonads also begin to develop at this time, starting as a single lobe near the gonadopore. They grow ventrally from the gonadopores and contain some ripe gametes when the urchin reaches a diameter of 6 mm, and an age of four to five months.

*Culture method:* The nutritional requirements of the urchin seem to be more complex or more restricted than those of the larva and it has taken more than a year to find an appropriate algal food that could be reliably cultured in the laboratory. At present a nonsterile surface dwelling diatom is used. This has been identified as a species of *Nitzschia.* Whether or not any of the bacteria in the culture are necessary, has not been determined. Cultures of this algae are maintained in Guillard's media.

The urchins are raised in plastic petri dishes (100 or 150 mm × 25 mm). The algae is first grown in these dishes until the bottom is covered (lightly for young urchins, heavier for older urchins), then the medium is diluted 50% with sea water and the urchins introduced. The dishes are kept in an illuminated incubator at 22–24° C. Under these conditions the algae growth tends to counterbalance consumption. Conditions for a balanced ecology are hard to establish

and ultimately either the algae is consumed or it grows so thick it begins to die off. In either situation the urchins are transfered to a fresh dish. If the young urchins are properly maintained they increase in diameter at a rate of approximately one millimeter in 18 days.

No real attempt has been made to determine urchin survival rates. This is because most of the urchins now being raised have been subjected to some experimental treatment. However, a rough estimate, based on experience, can be made. Survival depends, among other things, on the control of disease, maintenance of proper feeding conditions and on the particular male and female the gametes come from. Some matings appear to yield a hardier line of urchins than do others. When all the variables are taken into account, including survival of the plutei, somewhere around 50% of the young plutei can probably be grown to mature urchins. As more crosses are made within the laboratory stock, survival should improve.

Since some mature gametes can be obtained from urchins about four or five months old, a second generation can then be started. Therefore, the generation time is six months or less. Of course, no more than a few hundred eggs can be obtained at this stage, but these should be enough for genetic tests, or to establish a particular genetic line. Six months is significantly longer than the generation time of *Drosophila* but not so different from corn or mice, both of which have been used extensively in genetic studies. Therefore, if urchins are susceptible to genetic analysis, it should now be both possible and practical to use genetic techniques in the study of sea urchin development.

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## Summary

A method is described for raising sea urchins from egg to egg in the laboratory. The larvae are raised on flagellated marine algae and the young urchins on a substrate-dwelling diatom. The major features in the developmental process are: growth of the larva, development of the urchin inside the larva, metamorphosis and growth of the young urchin to sexual maturity. The entire life cycle takes about six months.

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